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Improvement of the quality of insect-cell-derived, recombinant pro-VEGF-C

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Tämän insinöörityön tarkoituksena oli parantaa hyönteissolujen tuottaman, rekombinantti pro-VEGF-C:n laatua. Korkealaatuista pro-VEGF-C:tä tarvitaan biolääketieteellisessä tutkimuksessa ja toistaiseksi sitä on saatavana vain rajallisissa määrin nisäkässolujen tuottamana. VEGF-C on viestimolekyyli, joka on välttämätön imusuoniston kasvulle. Sillä on myös keskeinen rooli monien sairauksien muodostumisessa ja siksi VEGF-C:n toimintaan kohdistettuja lääkkeitä kehitetään jatkuvasti.

Työ aloitettiin lisäämällä valmiiseen hyönteisekspressiovektoriin ampisilliini ja hygromysiini antibioottiresistenssigeenit yhdistelmä-DNA-tekniikalla, ja näin saatiin aikaan uusi vektori, pIZ-HygroA. PCR:llä monistettujen proteiinien calreticulinin, CDC37:n, PH4B:n, PDIA3:n ja syklofiliini B:n cDNA:t kloonattiin ensin TOPO vektoriin, jonka kautta uuteen pIZ-HygroA vektoriin. Nämä kyseiset proteiinit valittiin siksi, että niillä on ominaisuuksia, joiden oletetaan auttavan VEGF-C:n laskostumisessa. Uudet ekspressiovektorit transfektoitiin VEGF-C:tä tuottaviin hyönteissoluihin, joissa yllämainittujen auttajaproteiinien, sekä VEGF-C:n ilmentämistä ja laatua analysoitiin Western blot -menetelmällä, sekä bioanalyysillä, jolla mitataan VEGF-C:n aktiivisuutta.

Tutkimus saatiin suoritettua calreticulinin ja cyclophilin B:n osalta. Western blot -tulosten perusteella ei nähty eroa kovalenttisesti ja ei-kovalenttisesti sitoutuneiden VEGF-C dimeerien suhteessa ja hygromycin selektio näytti vaikuttavan alentavasti hyönteisolujen VEGF-C:n ekspressiotasoon. Ba/F3-VEGFR-3/EpoR-analyysissä kuitenkin nähtiin, että cyclophilin B:llä oli lievä vaikutus VEGF-C:n laatuun. Calreticulinilla vaikutusta ei havaittu. Tulosten varmistamiseksi, sekä tutkimuksen suorittamiseksi myös CDC37:llä, PH4B:lla ja PDIA3:lla tarvitaan kuitenkin vielä lisää tutkimuksia.

Avainsanat	VEGF-C, proteiinien laskostuminen, hyönteissolut, rekombinanttiproteiini, kloonaus

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The aim of this thesis work was to improve the quality of recombinant pro-VEGF-C produced by insect cells. High-quality pro-VEGF-C is needed for biomedical research and has been so far only available in limited amounts from mammalian expression systems. VEGF-C is a signaling molecule essential for the growth of lymphatic vessels. It plays important roles in many diseases and, therefore, many drugs are being developed, which target VEGF-C.

An existing insect cell expression vector was modified by adding antibiotic resistance genes for Ampicillin and Hygromycin B via restriction enzyme cloning, resulting in the novel vector pIZ-HygroA. cDNAs of calreticulin, CDC37, PH4B, PDIA3 and cyclophilin B were amplified by PCR and cloned into pIZ-HygroA via a TOPO intermediate. These proteins were chosen based on their presumed ability to assist in the folding of VEGF-C. Expression vectors were transfected into VEGF-C-expressing insect cells. The expression of helper proteins and VEGF-C expression levels and quality were analyzed by Western blotting and a bioassay, which measures VEGF-C activity.

The analysis was completed for calreticulin and cyclophilin B. The Western blots showed no difference in the ratio of covalently to noncovalently linked VEGF-C dimers. However, the process of hygromycin selection seemed to lower the VEGF-C expression levels of the insect cells. The results from the Ba/F3-VEGFR-3/EpoR assay indicated that cycophilin B might have a modest effect on the quality of VEGF-C, while calreticulin had no effect. Further analysis is still needed to confirm the results and to complete the analysis for CDC37, PH4B and PDIA3.

Keywords	VEGF-C, protein folding, insect cells, recombinant protein, cloning

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Abbreviations

ADP	Adenosine diphosphate
ATP	Adenosine triphosphate
BSA	Bovine Serum Albumin
CHO	Chinese Hamster Ovary –cell line
CIP	Calf Intestinal Phosphatase
DMEM	Dulbecco's Modified Eagle's Medium
dNTPs	Nucleotide triphosphate
DNA	Deoxyribonucleic acid
ECL	Enhanced chemiluminescence
ER	Endoplasmic reticulum
HighFive	Insect cell line originated from the ovarian cells of cabbage looper, *Trichoplusia ni*
HRP	Horseradish peroxidase
LB	Luria Broth
PBS	Phosphate Buffer Saline
PCR	Polymerase Chain Reaction
PDGF	Platelet derived growth factor
PDI	Protein disulfide isomerases
PlGF	Placental growth factor
RTK	Receptor tyrosine kinase
S2	*Drosophila* Scneider 2 Cells, a cell line that was derived from embryos of 20-24 hours old *Drosophila melanogaster.*
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis

Sf9	Traditional cell line used with baculovirus, derived from the pupal ovarian tissue of the fall army worm *Spodoptera frugiperda*
TBS	Tris Buffer Saline
VEGF	Vascular endothelial growth factor
VEGFR	Vascular endothelial growth factor receptor
VPF	Vascular permeability factor

1 Introduction

This thesis was done for the University of Helsinki, and the research work was conducted in the Jeltsch Laboratory (Translational Cancer Biology Program, Research Programs Unit) at the Medical Faculty. The aim of this thesis was to improve the quality of insect-cell-derived, recombinant pro-VEGF-C (Vascular Endothelial Growth Factor C). VEGF-C is a secreted glycoprotein, which induces the sprouting of new lymphatic vessels (lymphangiogenesis) and blood vessels (angiogenesis) from the existing vasculature. Because growth factors that stimulate lymphangiogenesis can promote the metastatic spread of cancer through the lymphatic system, VEGF-C is also a cancer drug target. Further research might reveal potential new targets in the VEGF system for new treatments of cancer and ischemic diseases. (Duong et al., 2012)

The expression of pro-VEGF-C has already been established in the insect-cell system; for example, via the baculovirus system or in transfected *Drosophila* cells *(S2).* However, VEGF-C features multiple intra- and intermolecular cysteine bonds. Different from mammalian cells, expression in insect cells can lead to a significant proportion of wrongly folded proteins, including incomplete and incorrect cysteine bonding. To avoid these problems, other expression systems such as the CHO (Chinese Hamster Ovary) overexpression system have been created for the expression of human proteins. Compared to, for example, the S2 cells, expression in CHO cells yields superior protein quality, but mammalian cells are generally more laborious and expensive to maintain and typically have lower yields compared to insect cells. (McCarroll and King, 1997)

Hence, the aim of this thesis was to improve the VEGF-C folding and cystine bonding pattern in the insect cell line Sf9. This was done by co-expressing VEGF-C together with five different, selected cDNAs whose translation products are involved in protein folding and disulfide bond formation. These five genes were Calreticulin (CALR), Hsp90 cochaperone (CDC37), Proline 4-Hydroxylase Beta (PH4B), Protein Disulfide Isomerase Family A, Member 3 (PDIA3), and PeptidyProlyl Isomerase B/Cyclophilin (PPIB).

2 Literature Review

2.1 Recombinant proteins

The production of recombinant proteins has become increasingly important as the demand for larger quantities of high-quality proteins has become commonplace in modern science. From a commercial view, also low costs and more efficient production are desired. The increasing knowledge and expertise to genetically manipulate organisms has led to significant improvements in recombinant protein production. Notably, an increasing fraction of all drugs being developed are based on recombinant protein technology. (Jarvis, 2015; Palomares et al., 2004)

2.2 Production of recombinant proteins in insect cells

The intended use of the protein is critical in choosing the expression host for producing recombinant proteins. Other factors to be considered are the molecular weight of the protein, the existence and amount of disulfide bonds (redox state), the destination of the produced protein (secreted versus intracellular) and different types of required post-translational modifications. For more complex proteins that require comprehensive post-translational modification, insect cells are often the host of choice since they are a good compromise between mammalian and bacterial systems. In insect cells, post-translational modifications happen in a very similar way as they do in mammalian cells. For example, for a secreted protein, signal peptides are cleaved and disulfide bonds are formed in the endoplasmic reticulum (ER) similar to mammalian cells, and similar enzymes are present for proteolytic processing. A major difference is, however, the N-glycosylation, where oligosaccharides (N-glycans) are added to asparagine side chains and then processed. N-glycans produced by mammalian cells feature prominently terminal sialic acid, whereas the N-glycans in insect cells have mostly terminal mannose residues. (Harrison and Jarvis, 2006). An additional advantage of insect cell cultures is their easy handling; no CO_2 regulation is needed and cells can be grown in shaking or spinner flasks. (Brondyk, 2009; Kollewe and Vilcinskas, 2013)

2.3 Improvement of the quality of insect cell derived recombinant proteins

N-glycosylation is one of the most typical post-translational modifications for secreted eukaryotic proteins. It plays an important part in the folding and quality control of proteins influencing the yield. The intended application of the recombinant protein often determines the requirements for glycosylation. Much effort has been made to modify the N-glycosylation of proteins in insect cells; mostly by introducing genes that encode the missing enzymes that are needed for a more mammalian-like N-glycosylation.

Another potential problem for the quality of secreted insect cell-derived protein is the capacity overload of the protein folding machinery in the ER. Expression levels in insect cells are typically higher than in mammalian cells. The amount of helper proteins (foldases, chaperones, isomerases) that is available might, therefore, not be enough for proteins that require extensive assistance to reach their correct conformation. These problems can be addressed by introducing the specific proteins needed.

In the baculovirus system, the helper proteins can be introduced into insect cells by co-infection, by constructing dual expression baculoviral shuttle vectors or by modifying the baculovirus genome. Alternatively, insect cells can be stably transformed with expression vectors for the proteins. The latter method was used in this project. In this method, DNA plasmid constructs are engineered to express the cDNAs of the helper proteins under the control of promoters, which are active in the specific insect cell lines. These DNA constructs are transfected into the insect cells and after integration into the insect cell genome a new cell line with enhanced protein production machinery is created. (Harrison and Jarvis, 2006)

2.4 Helper proteins

For this thesis work, five helper proteins were chosen for their abilities to act as chaperones or isomerases during the folding process of proteins. All chosen helper proteins are introduced in more detail in the chapters below.

2.4.1 Calreticulin (CALR)

Calreticulin was first discovered in 1974 and it has many functions in eukaryotic organisms but not in prokaryotes. First it was discovered in the ER, but since then it has been found to be a more widely distributed protein. It has been found in many other

cellular structures such as the cell membrane and cytoplasm. Calreticulin has two main functions: Maintaining calcium levels and acting as a chaperone to help proteins fold properly. In the ER, calreticulin functions as a molecular chaperone preventing aggregation of proteins that are only partially folded and increasing the amount of properly folded proteins by gathering more enzymes that take part in the folding. Calreticulin may interact with a protein several times as incorrectly folded proteins are re-glycosylated and interact with calreticulin again. (Mendlovic and Conconi, 2010; Michalak et al., 1999)

2.4.2 Hsp90 co-chaperone CDC37 (CDC37)

Hsp90 (Heat shock protein 90) is a molecular chaperone that has multiple functions in regulating cellular processes such as controlling cell cycle and survival and hormone signaling. It controls proper formation and activation of hundreds of different proteins referred to as “clients”. In addition to its presence inside cells, the Hsp90 has also been found on the cell surface and extracellular space which suggests a distinct extracellular chaperone activity. Hsp90 requires multiple co-chaperones in the chaperone cycle and CDC37 is one of them. It works in cooperation with Hsp90 but has also been shown to function independently. In the absence of Hsp90, recombinant CDC37 has intrinsic chaperone activity that resembles Hsp90, but the reaction is more efficient when both are present. It interacts specifically with kinases stabilizing them and keeping unfolded substrates in a competent state for other chaperones to refold them. (Li et al., 2012; MacLean and Picard, 2003)

2.4.3 Proline 4-Hydroxylase Beta (PH4B)

P4HB was first identified as the beta subunit of prolyl-4-hydroxylase (P4H), and it also is the first defined member of the protein family protein disulfide isomerases (PDI). All members of the PDI family have important roles in protein folding, and they are primarily expressed in the ER. P4HB has been found in almost all tissues and accelerates the oxidation and reduction of disulfide bonds. In addition, it has been found to have activity as a chaperon for nascent proteins. (Galligan and Petersen, 2012)

2.4.4 Protein disulfide isomerase family A, Member 3 (PDIA3)

PDIA3 was first called phospholipase C alpha and it is a member of the same PDI family as P4HB. PDIA3 is expressed in placenta, sperm, lungs, kidneys, heart, brain and skeletal muscle. It exists in the ER, nucleus, extracellular matrix and the plasma membrane. It is a chaperone that takes part in folding of N-glycosylated integral membrane proteins. It has been shown to participate in glycoprotein folding when attached to a complex that contains calreticulin and calnexin (Chen et al., 2010; Galligan and Petersen, 2012)

2.4.5 Peptidyprolyl isomerase B/Cyclophilin B (PPIB)

The cyclophilins are peptidylpropyl cis-trans isomerases (PPIases) that function as intracellular receptors. Another role for them is working as a chaperone for trafficking of proteins and macromolecular assembly. They take part in early folding of carbonic anhydrase by binding to hydrophobic domains that are exposed and thus preventing aggregation. Cyclophilin B can be found in the ER and in complexes on the plasma membrane. (Price et al., 1994)

2.5 Growth Factors

Growth factors are secreted, extracellular polypeptides that control growth, survival, differentiation and proliferation of cells in multicellular organisms. Controlling the proliferation of cells is important for multicellular organisms: cells should only divide when it is required to allow either for growth or to replace lost cells. Failing to regulate the behavior of individual cells is fundamental, for example, in the development of cancer. Over twenty different families of growth factors have been identified and the vascular endothelial growth factor family is one of them. (Alberts et al., 1998; Barret et al., 2010)

2.6 Vascular Endothelial Growth Factors (VEGFs)

The vascular endothelial growth factors belong together with the PDGFs (Platelet-derived growth factors) and a few other families into the cystine-knot super-family of hormones and signaling molecules. In all members of this super-family, six cysteine residues form a cystine-knot (one disulfide bond passes through a loop, which is

formed by the two other disulfide bonds). In vertebrates the VEGFs signal via receptor tyrosine kinases to induce angiogenesis, lymphangiogenesis and vessel permeability. They also play an important part in controlling vasculogenesis during embryonic development and in adults (Holmes and Zachary, 2005; Neufeld et al., 1999; Roskoski, 2007). The VEGF family consists of: VEGF-A, Placental growth factor (PlGF), VEGF-B, VEGF-C, VEGF-D, orf virus VEGF (VEGF-E) and VEGF-F (Holmes and Zachary, 2005; Roskoski, 2007). All VEGFs are composed of two antiparallel monomers that form a dimer. The dimer is mostly linked by two interchain disulfide bridges, but for VEGF-C and VEGF-D, both covalent and non-covalent dimers occur. Either two identical monomers (homodimer) or two different monomers can form a dimer (heterodimer). All VEGFs become N-glycosylated during biosynthesis except for VEGF-B, which is O-glycosylated for its 186 amino acid splice variant and completely unglycosylated for its 167 amino acid splice variant. (Hoeben et al., 2004)

2.6.1 VEGF-A

VEGF-A (also known as VEGF) was first discovered in 1983. It was first called vascular permeability factor (VPF) for its potential to enhance microvascular permeability. Later however, it was found to stimulate cell proliferation of endothelial cells and the name VEGF took over (Holmes and Zachary, 2005; Roberts and Palade, 1995). VEGF-A works as a mitogen and survival factor for vascular endothelial cells. Both of these features are needed for angiogenesis. Studies of VEGF-A show that it is a crucial factor during organ development and growth, and the disruption of the VEGF-A gene in these stages affects organ development, reduces growth and is finally lethal. VEGF-A-driven angiogenesis has also been found to have major roles in many human diseases such as cancer or rheumatoid arthritis. The VEGF-A gene has eight exons and produces nine alternative isoforms by alternative splicing of its pre-RNA, which result in differently sized proteins of 206, 189, 183, 165, 162, 148, 145 and 121 amino acids, the predominant form being the 165 amino acid isoform (VEGF-A_{165}). All variants contain exons 1-5 and 8, and the differences are generated by alternatively splicing exons 6 and 7. VEGF-A is composed of two antiparallel monomers that form a homodimer with two intermolecular disulfide bridges. It is expressed in most organs of the embryo, and after birth its expression generally declines. In adults, VEGF-A is mostly present in highly vascularized organs like the brain, lungs, kidneys and heart. Its expression increases during pregnancy, wound healing and tissue repair, but also in diseases that are asso-

ciated with neovascularization. VEGF-A has two receptors on endothelial cells: VEGFR-1 and VEGFR-2 (Holmes and Zachary, 2005; Roskoski, 2007).

2.6.2 PlGF

VEGF-A is the closest relative to PlGF with 42 % identity on the amino acid sequence level. This factor is (like VEGF-B) a VEGFR-1 specific ligand. Alone, it is only weakly angiogenic, but as a PlGF/VEGF-A heterodimer it is able to bind to VEGFR-2, which makes it mitogenic for blood vascular endothelial cells and hence angiogenic. Human PlGF exists in four splice isoforms: PlGF-1, -2, -3 and -4. PlGF-1 has 131, PlGF-2 152, PlGF-3 203 and PlGF-4 224 amino acid residues. PlGF is mostly expressed in the placenta, but also heart, retina, skin and skeletal muscle express various isoforms of PlGF. (Holmes and Zachary, 2005; Roskoski, 2007)

2.6.3 VEGF-B

The VEGF-B gene contains seven exons and gives rise to two isoforms through alternative splicing: VEGF-B_{167} and VEGF-B_{186}. Both isoforms activate only VEGFR-1. In mouse embryos, VEGF-B has been found in the heart and central nervous system; and in adults, it is mostly expressed in brain, heart, kidneys and testes; but some has also been detected in the liver, lungs and spleen. The role of VEGF-B has not been clearly identified yet, but it seems to have some special function for the adult heart. It does not seem to be required for angiogenesis or cardiovascular development. (Roskoski, 2007; Zhang et al., 2009)

2.6.4 VEGF-C

The VEGF-C gene features seven exons. Before secretion, VEGF-C undergoes proteolysis, which cleaves between the central VEGF homology domain and its C-terminal propeptide. After secretion, VEGF-C undergoes additional proteolytic processing to produce the mature forms of VEGF-C. These mature forms have (in addition to the eight canonical cysteine residues of the VEGF homology domain) one extra cysteine, which can interfere with inter-subunit disulfide bonding and result, depending on expression level and system, in a significant proportion of non-covalent VEGF-C dimers

and monomers (Figure 1). Studies show that splice isoforms for VEGF-C exist, but their functions are not known. (Holmes and Zachary, 2005; Krebs and Jeltsch, 2013)

VEGF-C is expressed in human adults in the heart, ovary, placenta, testes, small intestine and skeletal muscles. It has also been found in lower amounts in spleen, prostate, pancreas, lungs and kidneys. The main task of VEGF-C is the stimulation of lymphangiogenesis. Its unprocessed form binds only to VEGFR-3, which is the main driver of lymphangiogenesis, but the mature form binds also with high affinity to VEGFR-2, which gives it, in addition to its lymphangiogenic features, angiogenic potential. (Holmes and Zachary, 2005; Krebs and Jeltsch, 2013; Roskoski, 2007)

Because of the limited availability of pro-VEGF-C, almost all research on VEGF-C as a protein has been done with the mature, active form of VEGF-C (Jeltsch et al., 2006; Karkkainen et al., 2004; Kärpänen et al., 2006). However, in-vivo, VEGF-C is produced as a precursor (Figure 1). Recently, it has been shown that this precursor is not only incapable of activating its receptor, but that it acts as a competitive inhibitor of the mature, active VEGF-C (Jeltsch et al., 2014). For many experiments, the use of recombinant mature, active VEGF-C is therefore too simplistic and the use of pro-VEGF-C would resemble more the in-vivo situation.

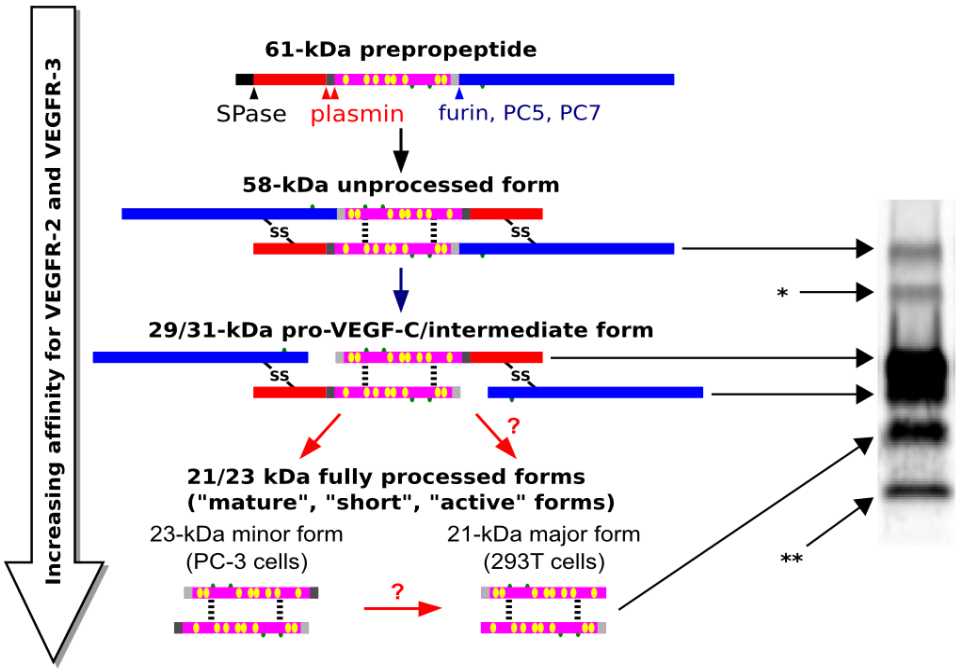

Figure 1. Enzymatic maturation of VEGF-C. (Krebs and Jeltsch, 2013)

2.6.5 VEGF-D

VEGF-D is also known as c-*fos*-induced growth factor (FIGF). It is structurally and functionally closely related to VEGF-C and also stimulates lymphangiogenesis. It binds to the same VEGF receptors and is processed into mature forms in a very similar way to VEGF-C. (Holmes and Zachary, 2005; Krebs and Jeltsch, 2013) However, unlike VEGF-C, it is not necessary for embryonic lymphangiogenesis in mice (Baldwin et al., 2005).

2.6.6 VEGF-E

VEGF-E denotes viral proteins that are homologous to VEGF. They have been found from different strains of the *Orf* parapoxvirus. VEGF-E appears to have an important role in the viral life cycle supporting angiogenesis in the virus-induced lesions. It has a permeability-increasing activity similar to VEGF-A. It binds to VEGFR-2 with high affinity, but not or only to a much lesser degree to VEGFR-1. (Holmes and Zachary, 2005; Roskoski, 2007)

2.6.7 VEGF-F

VEGF-F refers to proteins that are homologous to VEGF and have been discovered as components of snake venoms. Their presumed function is the amplification of the venom effect by increasing the permeability of blood vessels. (Krebs and Jeltsch, 2013)

2.7 Growth factor signalling

2.7.1 Receptor tyrosine kinases

Most growth factors act locally as mediators between neighboring cells (paracrine signaling) and responses require multiple intracellular steps to generate changes in cell behavior. Many growth factors work through receptors of the receptor tyrosine kinase (RTK) family. RTKs work by phosphorylating tyrosine residues in target proteins which in turn activate other proteins that induce, for example, cell growth and differentiation. (Alberts et al., 1998) RTKs consist of an extracellular ligand binding domain, a transmembrane domain and a cytosolic domain with protein-tyrosine kinase activity. There

are different mechanisms how RTKs can be activated. For the VEGF receptors, activation is triggered by dimerization of the receptor, which is caused by (bivalent) ligand binding to the extracellular domain. This induces the protein kinase in each monomer to phosphorylate a specific set of tyrosine residues in the intracellular domain of its partner. This process of cross-phosphorylation is referred to as autophosphorylation (depicted in Figure 2), and it enables other cytosolic signaling proteins to interact with the receptors and to forward the signal inside the cell into direction of the nucleus. (Lodish et al., 2000; Solunetti, 2006)

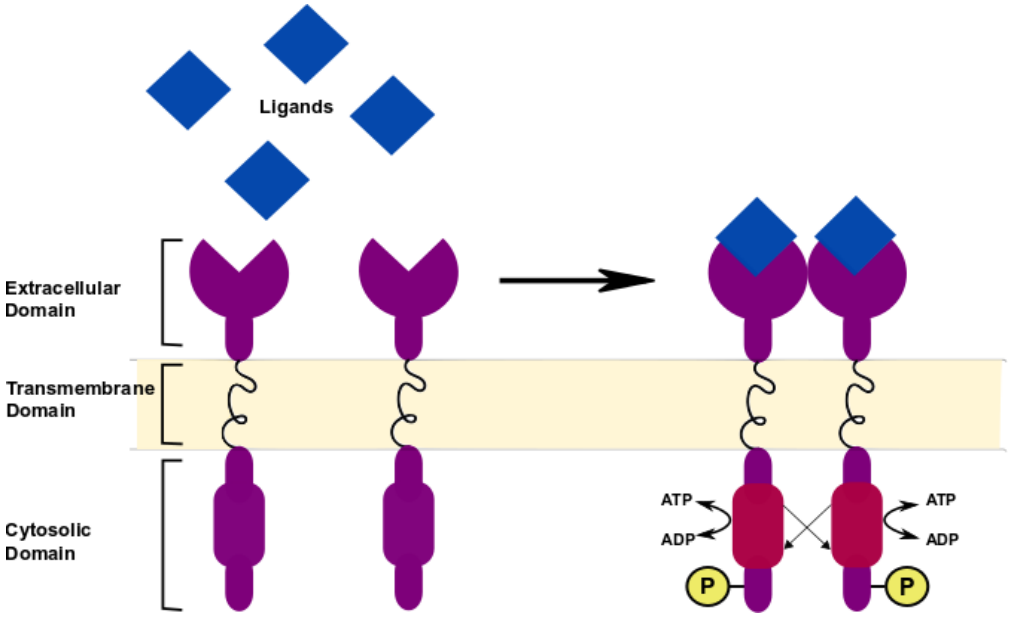

Figure 2. Schematic explanation of the RTK activation.

2.7.2 VEGF signalling

For the VEGFs, there are three receptor protein tyrosine kinases: VEGF-Receptor-1 (Flt-1), VEGF-Receptor-2 (Flk-1/KDR) and VEGF-Receptor-3 (Flt-4). They consist of an extracellular part that has seven immunoglobulin-like domains, a trans- and juxtamembrane segment, an intracellular split protein tyrosine kinase domain and a C-terminal tail (Roskoski, 2007). Different VEGFRs bind to specific VEGFs: VEGFR-1 binds to VEGF-A, VEGF-B and PlGF, VEGFR-2 binds VEGF-A, VEGF-C and -D and VEGF-E and VEGFR-3 binds VEGF-C and -D. Activation of each receptor leads to different outcomes as VEGFR-2 induces angiogenesis and vascular permeability and VEGFR-3 specifically regulates lymphangiogenesis. (Shibuya and Claessonwelsh, 2006) The function of VEGFR-1 seems to be context-dependent, but at least during early embryogenesis it negatively regulates angiogenesis (Fong et al., 1995).

2.8 Vasculogenesis

The functions of the vascular system are to supply tissues with nutrients and to dispose of waste products. The system extends into almost all organs and tissues and allows the exchange of small molecules, such as gases, nutrients and waste products. (Nagy et al., 2008) The formation of the vascular system consists of two important processes: vasculogenesis and angiogenesis. (Gale and Yancopooulos, 1999; Holmes and Zachary, 2005). Vasculogenesis is defined as the development of new blood vessels by differentiation of precursor cells. It characterizes the first phases of vascular development: the precursors of endothelial cells go through differentiation, expansion and coalescence forming primitive, homogeneously sized vessels (capillary plexus). (Gale and Yancopooulos, 1999)

2.9 Angiogenesis

The term angiogenesis refers to the sprouting of new capillaries from pre-existing vasculature. This process comprises the sprouting, splitting, branching and differentiation of blood vessels into the matured vasculature of adult organisms. In the adult, angiogenesis is generally needed during tissue growth, e.g. during pregnancy or wound healing. It also occurs as a contributing factor in many diseases such as cancer, rheumatoid arthritis, diabetic retinopathy and psoriasis. (Gale and Yancopooulos, 1999; Holmes and Zachary, 2005)

2.10 Lymphangiogenesis

The function of the lymphatic vessels is to transport tissue fluids, plasma proteins and cells back to the blood circulation. They act as tissue fluid removal system and also have an important part in immunological surveillance of the body. Lymphangiogenesis refers to the growth and formation of lymphatic vessels. During the embryonic development the lymphatic system starts to develop much later than the blood vascular system and begins after the cardiovascular system is already functional. In the beginning, migrating endothelial cells from the cardinal veins form the first structures of the lymphatics: the primary lymph sacs. From these, the lymphatic vessels start to grow by endothelial budding and sprouting, forming capillaries in most organs and tissues. In adults, physiological lymphangiogenesis is uncommon, but happens, for example, during the menstrual cycle and pregnancy. However, pathologic lymphangiogenesis is

characteristic for many diseases. (Chachaj and Szuba, 2013; Jeltsch et al., 2003; Karkkainen and Petrova, 2000)

3 Overview of the Study

3.1 Objectives and strategy

The first part of the project was to create an expression vector, into which the five selected cDNAs could be cloned. Vectors with all desired properties (antibiotic resistances, promoter, protein tag) were not commercially available and had to be constructed. The description of the cloning process is given in Chapter 3.2, and a chart of the cloning history can be found in Appendix 1. The next step was to amplify the five genes with PCR and thereby introduce suitable restriction sites for cloning. All PCRs were done with Phusion polymerase (New England Biolabs, NEB) and Dynazyme II (Thermo Scientific) so that products with both blunt ends and TA overhangs could be obtained for two different TOPO Cloning vectors. TOPO vectors were used so that the amplified cDNA inserts could easily be multiplied for further use. Different types of TOPO vectors were used to see whether one of them would be superior to the other and to test the influence of prolonged TOPO vector storage on the functionality of the TOPO system.

Inserts and vectors were then prepared by restriction digestion, isolated, ligated and later transfected into VEGF-C-producing Sf9 insect cells to see how the five selected helper proteins affect the expression of VEGF-C. To analyze the results, Western blots and Ba/F3 assays were performed. All methods are described in more detail below in the Materials and Methods section and a flowchart of the project is depicted in Figure 3.

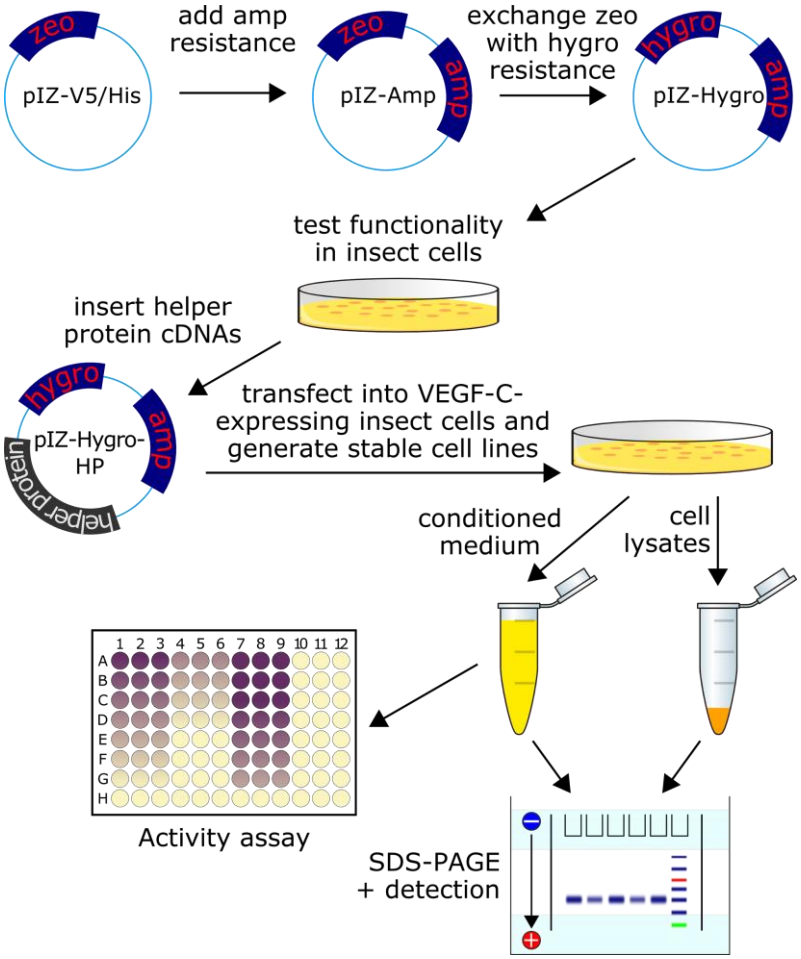

Figure 3. Flow chart of the project.

3.2 Cloning of the expression vector pIZ-hygroA

The research work of this thesis started by constructing a new expression vector for co-expressing VEGF-C with the five selected cDNAs. This was done by modifying the existing vector pIZ-V5/His (Invitrogen). An expression cassette for the ampicillin resistance gene was amplified by Polymerase Chain Reaction (PCR). The PCR product was phosphorylated with T4 polynucleotide kinase (NEB). The methylation state of the vector pIZ-V5/His was changed by subcloning it into the dam-negative *E.coli* strain in order to be able to use the restriction enzyme BspHI. After digestion, the vector was dephosphorylated with CIP (Calf Intestinal Phosphatase, NEB) to prevent recircularization. The insert was cut with enzyme AatI and overhangs from both the vector and the insert were blunted: they were either removed with T4 polymerase (3'-overhangs) or filled in with DNA Polymerase I, Large (Klenow) Fragment (5'-overhangs). Both vector and insert were then purified and extracted from an agarose gel using Nucleospin Gel and PCR clean-up kit (Macherey Nagel). An additional digestion with DpnI enzyme was

done on the insert to destroy residual template DNA from the PCR reaction. Insert and vector were ligated using a Rapid DNA ligation kit (Roche). The ligation products were transformed into competent *E. coli* and plated on Luria Broth (LB) ampicillin plates (10 g bacto-tryptone, 5 g bacto-yeast extract, 12 g bacto agar, 100 µg/ml ampicillin, ddH_2O ad 1 litre) and grown overnight. On the next day, colonies were picked, small-scale cultures grown overnight and plasmid DNA was isolated using a QIAprep Spin miniprep kit (Qiagen). To find out whether and in which direction the insert had been integrated into the vector, restriction analysis with DraI was performed on several clones. Clones containing the ampicillin resistance cassette insert in both orientations were replated on ampicillin plates, and an individual colony was picked and grown into a 200-ml-culture for DNA isolation. This construct was named pIZ-AmpA. The next step was to replace the zeocin resistance gene in pIZ-AmpA by a hygromycin resistance gene. The cDNA encoding for the hygromycin resistance gene was cut out from plasmid pCoHygro (Invitrogen) with restriction enzymes BamHI and BbvCI. The vector pIZ-AmpA was digested with PmlI and DraIII to remove the unwanted zeocin/GFP fusion gene. Its ends were blunted, dephosphorylated with CIP, and the fragment was purified by agarose gel electrophoresis. Ligation, transformation and plasmid DNA preparation ("minipreps") were done as described above. Restriction analysis was done with restriction enzymes BglI, EcoRI and PstI to determine presence and direction of the insert. One of the correct clones was chosen and plasmid DNA was isolated from a 200-ml-culture ("maxiprep"). The complete insert and junctional regions of this construct were sequenced (see Table 1), and it was named pIZ-HygroA.

Table 1. Primers used for sequencing pIZ-HygroA

Name	Sequense of the primer
V3448	5'-CAGACATGATAAGATACATTGATGAGTTTG-3'
M14866	5'-GTGGAACGAAAACTCACG-3'

The functionality of this construct (as in its ability to confer hygromycin resistance to insect cells) was tested by transfecting it into Sf9 cells using the Effectene transfection reagent (QIAgen). After transfection, cells were grown on a 24-well plate for 8 hours and the growth medium was changed. Two days later, hygromycin selection was started with Hygromycin B (Calbiochem) using different concentrations. The concentrations of hygromycin used were 15 µg/ml, 60 µg/ml, 250 µg/ml and 1000 µg/ml. All cells were maintained until the mock-transfected cells had died plus two weeks.

3.3 The genes of interest

Each of the five cDNAs (Calreticulin, CDC37, P4HB, PDIA3 and cyclophilin B) were amplified by PCR. Templates were: pCMV-Sport-6-hCALR (GenBank Acc. No. BC020493) for CALR, pOTB7-hCDC37 (GenBank Acc. No. BC000083) for CDC37, pDONR223-hP4HB (GenBank Acc. No. BC029617) for P4HB, pOTB7-hPDIA3 (GenBank Acc. No. BC014433) for PDIA and pOTB7-hPPIB (GenBank Acc. No. BC001125) for PPIB. All templates were provided by the Genome Biology Unit of the University of Helsinki (which hosts a partial replica of the Mammalian Gene Collection from the National Institute of Health). Phusion High Fidelity polymerase was used to create blunt-ended PCR products and Dynazyme II polymerase to create PCR products with adenine overhangs. Ready PCR products were purified from agarose gels using a NucleoSpin Gel and PCR clean-up kit. The inserts made by the Phusion High Fidelity polymerase were cloned into the Zero TOPO Blunt vector (pCR-Blunt II-*TOPO)* and the PCRs made by the Dynazyme II polymerase into the TOPO TA vector (pCRII-TOPO TA). The TOPO reactions were then transformed into competent *E. coli*, grown on LB-kanamycin plates overnight, and colonies were picked for DNA minipreps. The presence of the insert in the vector was confirmed by restriction analysis with EcoRI, and one or two clones were chosen for plasmid DNA maxipreps.

3.4 Cloning of the final constructs

Subclonings into the final constructs were made by conventional restriction cloning methods (restriction digest, gel purification, ligation and transformation to *E. coli*). The restriction enzymes used for each insert and vector are listed in Table 2.

Table 2. Restriction enzymes used for the cloning of the expression constructs.

	Insert	Vector
CALR	KpnI, NheI	KpnI, XbaI
CDC37	StuI, NheI	Eco32I, XbaI
P4HB	KpnI, NheI	KpnI, XbaI
PDIA3	StuI, XbaI	Eco32I, XbaI
PPIB	StuI, NheI	Eco32I, XbaI

All restriction digests were done sequentially with a buffer exchange in between using the NucleoSpin Gel and PCR Clean-up kit (PCR clean-up protocol). After ligation all cloning steps were done as described before. The PCR-derived parts of the constructs were sequenced, and each construct was transfected into VEGF-C-expressing Sf9 cells.

3.5 Analysis of the VEGF-C expression

For SDS-PAGE, 4-20 % gradient gels (Bio-rad) were used. Samples were loaded, and the proteins were allowed to migrate on the gels for a sufficient time. Proteins were then transferred from the gels to membranes. Membranes with the supernatant samples were blocked for one hour in blocking buffer (10% BSA (Bovine Serum Albumin), 0.2% Tween 20) and incubated overnight with primary antibody against VEGF-C in blocking buffer. Membranes were washed with TBS-T (0.1% Tween 20), incubated with secondary antibody and washed again. Enhanced chemiluminescence reaction (ECL) was done to detect the proteins with the Odyssey Fc (Li-Cor) device. For the membranes with the lysis samples, Odyssey Blocking Buffer, PBS (Li-Cor) was used, and primary antibodies were anti-V5 to recognize the V5 tag in the constructs and β-actin (one of the major components of the cell cytoskeleton). Infrared dyes were used to induce a fluorescence reaction to be detected with the Odyssey Fc wavelengths 700 and 800 nm.

In addition to Western blots, Ba/F3 assays were conducted. Ba/F3 cells were washed with fresh DMEM medium, divided on 96-well plates with Interleukin-3 (IL-3) and zeocin selection (125 µg/ml) Different dilutions of insect cell suspensions were added to each well, all in triplicates. To measure the effect the insect cell derived, recombinant VEGF-C had on the survival of the Ba/F3 cells, a color reaction with MTT (3-(4,5-dimethyl thiazolyl-2) -2,5-diphenyltetrazolium bromide) was carried out, and after lysing the cells, color densities were measured.

4 Materials and Methods

4.1 PCR

In this thesis project, two different PCR polymerases were used for the amplification of the genes of interest: Phusion polymerase and Dynazyme II polymerase. Two polymerases were needed because the inserts were cloned into TOPO-vectors from two different TOPO-kits: the Zero TOPO Blunt kit and the TOPO TA PCR Cloning kit. The Zero TOPO Blunt cloning kit is meant for the cloning of blunt-ended PCR products, and the proofreading Phusion HF polymerase creates PCR products with blunt ends. The TOPO TA cloning kit requires PCR products with 3'-adenine overhangs and the Taq and Dynazyme II polymerases both have a nontemplate dependent terminal transferase activity that adds a single deoxyadenosine (A) to the 3' ends of PCR products. All PCRs were done with Applied Biosystem's 2720 Thermal Cycler PCR machine. Compatible reaction buffers were used for both polymerases and PCR reactions were done in volumes of 100 µl. Table 3 shows the reaction mixes for both polymerases.

Table 3. Reaction mixes for PCRs.

Phusion HF Polymerase		Dynazyme II Polymerase	
5x HF Phusion Buffer	20 µl	10x Dynazyme Buffer	10 µl
10 mM dNTPs	2 µl	10 mM dNTPs	2 µl
fwd Primer (100 µM)	1 µl	fwd Primer (100 µM)	1 µl
rev Primer (100 µM)	1 µl	rev Primer (100 µM)	1 µl
Template DNA (390-3939 ng/µl)	1 µl	Template DNA (390-3939 ng/µl)	1 µl
Polymerase (2 U/µl)	1 µl	Polymerase (2 U/µl)	1 µl
H2O	72.9 µl	H2O	82.9 µl

Template DNAs were diluted so that each PCR reaction had 200 ng of template DNA, and the water used in every PCR was molecular grade (free of DNase, RNase and

protease contaminations). The primers used for each gene and the template sources are listed in Table 4. PCR conditions for the two different amplifications are listed in Table 5.

Table 4. Primers used in PCR for amplifying the genes of interest.

Gene	Primer number	Primer
CALR fwd	V929	5'-TCACACAGGAAACAGCTATGAC-3'
CALR rev	V5940	5'-GCGCTAGCAACAGCTCGTCCTTGGC-3'
CDC37 fwd	V1705	5'-CGATTTAGGTGACACTATAGAAC-3'
CDC37 rev	V5944	5'-GGGCTAGCAACACACTGACATCCTTCTCA-3'
P4HB fwd	V387	5'-CGCCAGGGTTTTCCCAGTCACGAC-3
P4HB rev	V5943	5'-GCGCTAGCAACAGTTCATCTTTCACAGCTTTCTG -3
PDIA3 fwd	V1705	5'-CGATTTAGGTGACACTATAGAAC-3'
PDIA3 rev	V5942	5'-GCTCTAGAAAGAGATCCTCCTGTGCCTTC-3'
PPIB fwd	V1705	5'-CGATTTAGGTGACACTATAGAAC-3'
PPIB rev	V5941	5'-GCGCTAGCAACTCCTTGGCGATGGC-3'

Table 5. PCR conditions for amplifying the genes of interest.

Phusion HF		Dynazyme II	
98°C	1 min	94°C	1 min
98°C	10 s	94°C	30 s
58°C	15 s	45°C	15 s
72°C	39 s	72°C	60 s
72°C	5 min	72°C	4 min

4.2 TOPO-Cloning

The TOPO cloning method is based on DNA topoisomerase I enzyme. This enzyme works as both restriction enzyme and a ligase. It binds to the double stranded DNA at specific sites and cuts the phosphodiester backbone of one DNA strand after the sequence 5′-CCCTT. Breaking of the backbone releases energy, which is preserved by forming of a covalent bond between the 3’ phosphate of the cut strand and a tyrosyl residue (Tyr-274) of the topoisomerase I. This bond between the enzyme and DNA can be attacked by the 5’ hydroxyl of the insert DNA releasing the topoisomerase. A schematic view of the topoisomerase I reaction in Figure 4.

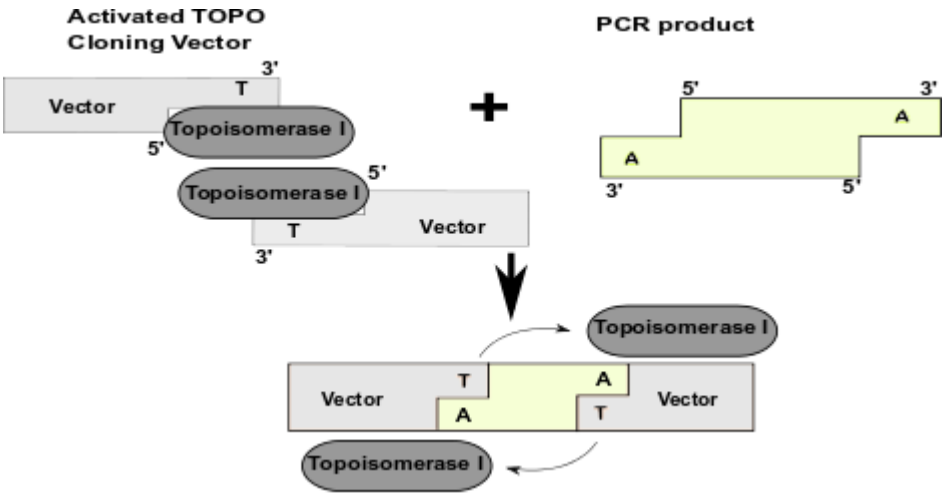

Figure 4. Topoisomerase reaction.

In the reaction mixture, the PCR product is mixed with the TOPO vectors and a salt solution (200 mM NaCl, 10 mM $MgCl_2$). Addition of salt prevents the topoisomerase I from rebinding and nicking the DNA after ligation of the PCR product and detaching it from the vector DNA. As a result more intact molecules are formed, and transformation efficiency is higher. Finally water is added. The reaction is done at room temperature and the incubation time is 5-30 minutes.

4.3 Transformation of vector to *E. coli*

Transformations were done into chemically competent NEB 5-alpha *E. coli*. The frozen cells were removed from -80°C and thawed on ice for 10 minutes. Aliquots of 50 µl were pipetted into 1.5 ml Eppendorf tubes. 2.5 µl of ligation or TOPO reaction mixture were added and the tubes were quickly flicked few times to mix the cells. The mixture was then incubated on ice for 30 minutes. After incubation, the cells were heat shocked at 42°C for 30 seconds and then placed back on ice for 5 more minutes after which 450 µl of room temperature SOC outgrowth medium (NEB) was added. The bacteria were then incubated in a 37°C bacterial shaker under vigorous agitation for one hour and then plated on Luria-agar plates with 50 µg/ml kanamycin.

4.4 Miniprep

“Miniprep” is a method for the purification of small amounts of plasmid DNA. For this thesis project the QIAprep Spin miniprep kit was used. Contents of the kit are as follows:

- QIAprep Spin Columns
- Resuspension buffer P1
- Lysis buffer P2 (NaOH, SDS)
- Neutralization buffer N3
- Binding buffer PB
- Wash buffer PE
- Elution buffer EB (10 mM Tris-Cl, pH 8.5)
- RNase A
- LyseBlue reagent
- Collection tubes and spin columns

In addition to these, ethanol (96–100%) was added to buffer PE and RNase A and LyseBlue reagent were added to buffer P1. This kits procedure has five steps: preparation and clearing of bacterial lysate, adsorption of DNA onto silica membrane and washing and eluting of plasmid DNA. First, an appropriate number of colonies were picked from plates and grown overnight in 5 ml Luria broth under antibiotic selection. Cells were then centrifuged 3000 g for 5 minutes to pellet the cells, and the supernatant was removed. After that the cells were then resuspended in 250 µl buffer P1 and moved to 1.5 ml Eppendorf tubes. A total of 250 µl of P2 buffer were added to lyse the cells, and the tubes were inverted 4-6 times until the solution turned blue. This kit lyses bacteria under alkaline conditions. SDS solubilizes the cell membrane's phospholipid and protein components, which lyses the cell and releases the cell content while chromosomal and plasmid DNAs and proteins are denatured under the alkaline conditions. The lysis reaction must not be allowed to proceed over 5 minutes or the DNA might become irreversibly denatured. Then, 350 µl of buffer N3 were added, and the solutions mixed by inverting the tubes 4-6 times until the solution turns cloudy and colorless. Buffer N3 neutralizes the lysate and adjusts the conditions for high salt binding to the spin column. The high salt concentration makes denatured proteins, cellular debris, chromosomal DNA, and SDS precipitate, while plasmid DNA renatures and stays in solution. The solution was centrifuged for 10 minutes at 13 000 g on a tabletop centrifuge (Eppendorf Centrifuge 5415 D) and the supernatant was applied to spin columns. These were centrifuged for 60 second at 13 000 g, and the flow-through was discarded. At the high salt concentration of the solution only plasmid DNA is adsorbed to the silica membrane of the spin columns while cell metabolites and cellular proteins remain in the flow-through. To remove endonucleases, 500 µl of buffer PB were added, columns were centrifuged again for 60 seconds at 13 000 g and the flow-through discarded. Next, salts were removed by adding 750 µl of buffer PE, centrifuging for 60 seconds at 13 000 g and removing the flow-through. To make sure that no washing buffer remained in the columns, they were centrifuged once more for 60 seconds and the columns were moved to clean 1.5 ml Eppendorf tubes. Finally, the elution of DNA was achieved by adding 50 µl of buffer EB to the center of the column beds, incubating the columns for 60 seconds and centrifuging them for 60 seconds at 13 000 g. Small aliquots, typically 5 or 10 µl, of the samples were digested with restriction enzymes as described in Chapter 4.5 and resolved on agarose gels to confirm the identity of the sample DNA by generating a specific band pattern.

4.5 Restriction analysis

Restriction enzymes were chosen for each construct individually. Some of the enzymes were purchased from NEB and some from Thermo Scientific. Buffers for each digest were chosen depending on the enzyme. For NEB enzymes, compatible buffers were chosen from among the following:

- NEB buffer 1 (10x concentrated, 10mM Bis-Tris-Propane-HCl, 10mM $MgCl_2$, 1mM DTT)
- NEB buffer 2 (10x concentrated, 50mM NaCl, 10mM Tris-HCl, 10mM $MgCl_2$, 1mM DTT)
- NEB buffer 3 (10x concentrated, 100mM NaCl, 50mM Tris-HCl, 10mM $MgCl_2$, 1mM DTT)
- NEB CutSmart buffer (10x concentrated, 50mM Potassium Acetate, 20mM Tris-acetate, 10mM Magnesium Acetate, 100 µg/ml BSA)

When using NEB buffers 1, 2 or 3, 10% (BSA, 1 µg/ml) was added. All enzymes from Thermo Scientific, except KpnI, were FastDigest enzymes and therefore compatible with FastDigest buffer. For KpnI (Thermo Scientific), KpnI Buffer with BSA was used (10 mM Tris-HCl, 10 mM $MgCl_2$, 0.02% Triton X-100, 0.1 mg/ml BSA) and for EcoRI (NEB), NEB EcoRI buffer (100 mM Tris-HCl, 50mM NaCl, 10 mM $MgCl_2$, 0.025% Triton X-100). Sterile H_2O was used for the reactions. The amount of DNA varied between 1.5 and 5 µg for each digest.

A typical volume for analytical restriction digests was 25 µl and for preparative digests 100 µl. An example of a reaction mix is shown in Table 6.

Table 6. Example of a restriction digest reaction mix.

DNA (1 µg)	2 µl
Buffer	2.5 µl
(BSA 1:10)	2.5 µl
Restriction enzyme	0.5 – 1.5 µl
H_2O	Added to final volume of 25 µl

In the preparation of vectors that were used for ligations, 1 µl Thermosensitive Alkaline Phosphatase (FastAP) was added for every µg of DNA after the restriction digest and incubated for 15 min at 37°C to prevent recircularization of the plasmids. Reaction mixtures were then loaded on agarose gels for analysis or purification.

4.6 Agarose gel electrophoresis

Most gels were 1% agarose gels made from Pulse Field Certified Agarose (Bio-Rad) and 1x TAE buffer (40 mM Tris, 20 mM acetic acid, 1 mM EDTA, pH 7.6). The agarose and TAE buffer were weighed and mixed and then boiled in a microwave oven for 2-3 minutes until all agarose was dissolved. The agarose solution was cooled to approximately 55°C, and ethidium bromide (10 mg/ml, Bio-Rad) was added (2-4 µl for 40-80 ml gel volume) into agarose to visualize the DNA with ultraviolet light. Then, the solution was poured into a mold with a comb for forming the sample wells and it was allowed to set for 30 minutes. After the gel had set, the comb was removed and the gel was placed in the electrophoresis chamber and covered with 1x TAE buffer. DNA samples with loading dye (6x concentrated, Thermo Scientific) were loaded onto the gel and an electric current was applied. As nucleic acids are negatively charged, DNA migrates towards the positive electrode on the gel and small weight molecules migrate faster than bigger ones. Lastly, the UV illuminated gel was photographed and the observed fragments sizes compared to the predicted sizes in order to identify correct clones (for analytical gels) or to identify the correct DNA fragments that need to be excised and isolated for cloning purposes. (Yılmaz et al., 2005)

4.7 DNA maxiprep

NucleoBond-Xtra Maxi kit (Machery Nagel) was used for DNA maxipreps in this thesis project. Maxiprep is a method for the purification of large amounts of plasmid DNA. Contents of the kit were as follows:

- Resuspension buffer RES (with RNase A added)
- Lysis buffer LYS
- Equilibration buffer EQU
- Washing buffer WASH
- Elution buffer ELU
- Columns and column filters

For this procedure, cells were grown at 37°C overnight in 200 ml LB under antibiotic selection and vigorous shaking. The cells were then centrifuged for 30 minutes at 6000 g and 4°C, and the supernatant was discarded. The pellet was resuspended in 12 ml RES buffer, and cells were lysed with 12 ml of LYS buffer. The solution was mixed by inverting 5 times and incubated at room temperature for 5 minutes. A total of 12 ml of neutralization buffer NEU were added. The neutralization buffer contains potassium acetate which causes the SDS in the lysis buffer to precipitate pulling down any cellular debris, chromosomal DNA and proteins. By neutralizing the lysate, the plasmid DNA returns into its super-coiled structure and remains in solution. The suspension was inverted until samples turned colorless. Columns with filters were placed on a rack, and the filters were equilibrated with EQU buffer, and the column was allowed to empty by gravity flow. The lysates were loaded into the column, and the column was again allowed to empty by gravity flow. The filters were washed with 15 ml of buffer EQU to make sure lysates were quantitatively entering the column, and then the filters were discarded. The columns were washed twice with 25 ml buffer WASH, and then the plasmid DNAs were eluted with 15 ml buffer ELU. To precipitate the eluted plasmid DNAs, 10.5 ml of room-temperature isopropanol were added; the solutions were mixed and then centrifuged for 30 minutes at 15 000 g and 4°C. The supernatants were discarded, and the DNA pelletes washed with 4 ml 70% ethanol. The tubes were again centrifuged (5 minutes at 15 000 g), the ethanol was removed and the pellet was dried for 15-30 minutes. Finally, the DNA pellet was dissolved in 500 µl buffer TE and the DNA concentration was measured spectrophotometrically.

4.8 Gel Purification

After PCR and restriction digestion, DNA fragments were purified by electrophoresis on 1% agarose gels. The extraction of DNA from excised gel pieces was performed with the NucleoSpin Gel and PCR clean-up kit (Macherey-Nagel). Contents of the kit were as follows:

- Binding buffer NTI
- Wash buffer NT3
- Elution buffer NE (5 mM Tris/HCl, pH 8.5)
- NucleoSpin Gel and PCR Clean-up columns and collection tubes

The DNA bands of right size were cut from the gel and weighed. 200 µl of buffer NTI were added for 100 mg of gel. Then, the samples were incubated 5-10 min at 50°C and mixed/vortexed every two minutes until the gel pieces had completely dissolved owing to the effects of the chaotropic salt in the binding buffer. The columns were then placed into the collection tubes, and the samples were applied to the columns. Similar to the plasmid DNA kits, the DNA binds under high salt concentration to the silica membrane of the columns. Then the columns were centrifuged for 30 seconds at 11 000 g, and the flow throughs discarded. To remove impurities, columns were placed back into the collection tubes and 700 µl of NT3 washing buffer were added, columns were centrifuged for 30 seconds at 11 000 g, and the flow-throughs were discarded. This washing step was done twice. To remove all residual washing buffer, the columns were centrifuged for 1 min at 11 000 g and then they were placed into clean 1.5 ml Eppendorf tubes. To elute the isolated DNA fragments, elution buffer NE was added 15 µl, samples were incubated for 1 min at room temperature and centrifuged for 1 min at 11 000 g.

4.9 Ligation of inserts and vectors

In the ligation reaction, the DNA ligase covalently joins the insert and the vector by catalyzing the formation of phosphodiester bonds. For the ligations of this work, the Rapid DNA ligation kit (Roche) was used. Contents of the kit were as follows:

- T4 DNA Ligation Buffer, 2x concentrated
- DNA Dilution Buffer, 5x concentrated
- T4 DNA Ligase

Ligations were done in volumes of 10 µl, and the molar ratio of insert to vector in each ligation was approximately 3:1. The vector and insert fragments were diluted into 1x concentrated DNA Dilution Buffer to a final volume of 5 µl. After adding 5 µl of T4 DNA Ligation Buffer, the reactions were mixed thoroughly and 1 µl of T4 DNA ligase was added to each reaction. The ligation reactions were incubated for 30 minutes at room temperature and then transformed into *E. coli*.

4.10 Transfection of pIZ-HygroA with helper protein cDNAs into insect cells

Transfections were done with the Effectene Transfection Reagent (Qiagen) according to the instructions of the manufacturer. Constructs were transfected into Sf9+VEGF-C cells that had been grown on 12-well plates using protein free Insect-Xpress medium (Lonza) with penicillin (100 units/ml), streptomycin (0.1 mg/ml) and 50 µg/ml of zeocin at 26°C without shaking. After transfection, cells were moved to 6-well plates after they had reached confluency. 60 hours after transfection, the selection with hygromycin was started. For each construct concentrations of 10 µg/ml and 20 µg/ml hygromycin were used. 50 µg/ml of zeocin were included for all cells to maintain selection for the VEGF-C expression. Untransfected cells were used as negative controls. Growth medium was exchanged twice weekly until cells were optically healthy, and no major signs of cell death could be detected anymore (4-5 weeks). The original cell line (Sf9+VEGF-C) was maintained for the same period at 50 µg/ml of zeocin.

4.11 Counting cell densities

Cell densities were counted with a Bio-Rad TC10 Automated Cell Counter. A total of 15 µl of suspended cells were pipetted into dual chamber cell counting slides (Bio-Rad) with 15 µl of trypan blue for staining. Trypan blue stains only dead cells leaving live cells with intact cell membranes unstained. The Bio-Rad TC10 Automated Cell Counter automatically counts the density of live cells as well as the percentage of live cells in the sample when the counting slide is inserted into the machine. Cells were counted in duplicates or triplicates due to the relatively large error of the counter, and the average cell density was calculated.

4.12 SDS-PAGE and Western blot

For SDS-PAGE, Sf9 cells from 6-well plates were resuspended by pipetting. The cell suspensions were transferred into 2-ml-tubes, and the cell densities were counted and adjusted to 0.5 Mio./ml with fresh medium after centrifugation (Eppendorf Centrifuge 5415D, 4 minutes, 400 g). 2.5 ml of cell suspension were plated to 6-well plates and cultured for three days (for Western blotting) or five days (for Ba/F3 assays). The culture for Western blotting was performed under zeocin selection, but without hygromycin B selection. The culture for the Ba/F3 assay was grown without any antibiotics.

After 3-5 days of culture, supernatants were harvested by repeated centrifugation (once as above and after transfer of the supernatant into a clean tube for 10 minutes at 16 000 g and 4ºC). Cells pellets from the first centrifugation step were lysed with 200 µl of lysis buffer for each sample. Contents of the lysis buffer (10 ml) were as follows:

- 150 mM NaCl
- 50 mM Hepes
- 1% NP-40
- 100 µl Leupeptin
- 50 µl Aprotinin
- 60 µl PMSF (phenylmethylsulfonyl fluoride)

After vortexing, lysis was allowed to proceed for 30 minutes at +4ºC under agitation. Samples were then centrifuged in a cold centrifuge (Eppendorf Centrifuge 5417R) for 5 minutes at full speed, and the supernatant was collected. Half of each sample was boiled after adding 5x concentrated reducing Laemmli buffer (60 mM Tris-Cl pH 6.8, 2% SDS, 10% Glycerol, 5% 2-Mercaptoethanol, 0,001% bromophenol blue) while the other half was boiled in non-reducing buffer (as above, but without 2-Mercaptoethanol). For the cell lysate samples, only reducing Laemmli buffer was used. 10 µl of the samples were loaded on 4-20% gradient SDS-PAGE gels (Bio-rad) with Pageruler Prestained protein ladder (Thermo Scientific). Gels were run until the smallest band of the molecular weight marker had reached the bottom of the gel limiting the voltage to 200 V and the current to 40 mA per gel to separate the proteins of the samples. The proteins were transferred to Immunomobilon-FL PVDF transfer membranes (Millipore) using a Transblot SD Semidry Transfer Cell (Bio-Rad) and Semi-dry transfer buffer (2 l buffer: 29.1 g Tris, 14.6 g Glycine, 250 ml Methanol, 9.4 ml 20% SDS, H_2O). Membranes with the supernatant samples were blocked for one hour in blocking buffer (10% BSA, 0.2% Tween 20) and incubated overnight with affinity-purified polyclonal goat antibody against VEGF-C (R&D Systems, AF752, 1:2000) in blocking buffer. Membranes were washed with TBS-T (0.1% Tween 20) 3 times for 5-10 minutes and secondary antibody, horseradish peroxidase (HRP)-conjugated polyclonal rabbit anti-goat immunoglobulins in blocking buffer were used (Dako, P0449, 1:2000). After 30 minutes of incubation, membranes were washed again and incubated with chemiluminescence substrate (Super Signal West Pico Chemiluminescence Substrate, Thermo Scientific) and detected using the Chemi function of the Odyssey Fc device. For the membrane with the lysate samples, blocking was done for one hour in the Odyssey Blocking Buffer (PBS) (Licor, #927-40000) and incubation overnight in the same buffer with the

mouse anti-V5 antibody (1.1 mg/ml, Invitrogen #46-0705, 1:6000 dilution) and the rabbit anti-β-actin antibody (Cell Signaling #4967S, 1:1000 dilution). After washing the membrane three times for 5-10 minutes with PBS-T (0.1% Tween 20), the membrane was incubated with the secondary antibodies (anti-mouse IRDye680 and anti-rabbit IRDye800, both 1:15000 in Odyssey Blocking Buffer (PBS)). After washing three times for 5-10 minutes with PBS-T, the proteins were visualized with the Odyssey Fc device using wavelengths of 700 and 800 nm.

4.13 Ba/F3 cell survival bioassay

In this assay, Ba/F3-VEGFR-3/EpoR cells were used. This cell line expresses a chimeric receptor consisting of the extracellular domain of VEGFR-3 and the intracellular domain of the erythropoietin receptor (EpoR). Cells were maintained in Dulbecco minimal essential medium (DMEM) with 10% fetal calf serum (FCS), supplemented with IL-3 and zeocin (125 µg/ml). These cells survive and grow only in presence of either IL-3 or VEGF-C. When IL-3 is not added to the culture medium, levels of active VEGF-C can be quantified by measuring cell survival. MTT is a substrate that is converted by mitochondrial activity of live cells into a purple colored product, which is measured at 540 nm.

To remove residual VEGF-C, cells were washed three times with DMEM medium. Washing was done by first centrifuging the cells for 4 minutes at 1200 g, removing the supernatant resuspending the cells in fresh media. Cell densities were counted, and the density adjusted to 0.4 Mio./ml. 50 µl of this cell suspension were aliquoted into each well of a 96-well plates (20 000 cells/well), into which different dilutions of the insect cell suspension in 50 µl DMEM had been pipetted in triplicates. Samples (conditioned insect cell medium) were used at concentrations of 0.27 to 20%. Purified, recombinant mature (active) VEGF-C (Kärpänen et al., 2006) was used as a positive control. To account for the growth-inhibiting properties of insect cell medium for the Ba/F3 cells, the recombinant VEGF-C was diluted in insect cell medium and used at concentrations of 0.27 to 20% (corresponding to final VEGF-C concentrations of 0.376 to 274 ng/ml).

Plates were incubated in a cell culture incubator for 48 hours in 37°C, after which 10 µl of MTT (5 mg/ml in PBS) were added to each well. Incubation was continued for another 2 hours after which the MTT reaction was stopped by adding 100 µl MTT lysis

buffer (20% SDS, 0.083% conc. HCl in H_2O). In order to dissolve the purple crystals and to be able to accurately measure the color density, the plates were incubated in the dark at 37°C overnight. Color density was measured with a spectrophotometer (Labsystems Multiscan Ascent) at 540 nm.

5 Results

5.1 Cloning of pIZ-AmpA and pIZ-AmpB

Introduction of the ampicillin expression cassette into the pIZ-V5/His vector was successful as the bacteria did grow on ampicillin plates. However, after replating individual clones, there was clear difference in the colony size between different clones. Restriction analysis of 6 clones (3 small colonies and 3 normal colonies) showed that colony size was associated with the orientation of the inserted ampicillin cassette. In the three clones that formed normal-sized colonies, the ampicillin cassette had been inserted into the same direction to the (existing) zeocin resistance gene (pIZ-AmpA) and in the 3 clones that grew only small colonies, the ampicillin cassette had been inserted in the opposite direction to the zeocin resistance gene (pIZ-AmpB). pIZAmpB colonies did not grow (or only very slowly) in a suspension culture under 100 µg/ml ampicillin. One clone of pIZ-AmpA was chosen for the further cloning.

5.2 Cloning and testing of the expression vector pIZ-HygroA

The coding sequence for zeocin/GFP was replaced in the pIZ-AmpA vector by the hygromycin coding sequence. From 12 analyzed clones, 10 contained the insert in the wrong orientation (pIZ-HygroA-INVERSE) and 2 clones contained the insert in the correct orientation with respect to the OpIE-1 promoter and SV40 polyA sequences. Clone number 7 (pIZ-HygroA), which was chosen for DNA sequencing of the hygromycin coding region and junctional sequences, showed exactly the predicted sequence.

The hygromycin resistance gene of pIZ-HygroA was functional in insect cells as judged by the ability of transfected Sf9 cells to tolerate well a hygromycin concentration of 15 µg/ml, whereas untransfected Sf9 cells died already after a few days under the same conditions. However, at all three higher hygromycin concentrations that were tested

(60, 250 and 1000 µg/ml), both transfected and untransfected Sf9 cells died rapidly within a few days.

5.3 Cloning of the genes of interest

Only two of the original five cDNAs were successfully cloned into the expression vector: the cDNAs for calreticulin and cyclophilin B. The calreticulin cDNA was subcloned from the TOPO Blunt vector as a KpnI/NheI fragment into KpnI/XbaI-opened pIZ-HygroA and the cyclophilin B from the TOPO T/A vector as a StuI/NheI fragment into Eco32I/XbaI-opened pIZ-HygroA. DNA sequencing confirmed the correct inserts and that no PCR errors had been introduced. The cDNA for CDC37 (Mammalian Gene Collection, IRAU plate 14, a 6) that was obtained from the Genome Biology Unit at the University of Helsinki appeared to be a wrong construct (HMGA1 cDNA) as determined by the inability to amplify the CDC37 sequence with specific primers and by DNA sequencing. A replacement clone was obtained (Mammalian Gene Collection, IRAU 31 b 1), which was PCR-amplified and cloned into the TOPO T/A vector, but it was too late to include it in further experiments.

The TOPO T/A cloning reaction for the P4HB and the PDIA3 cDNAs yielded only very few colonies. DNA minipreps were prepared for all of them, but DNA sequencing did not reveal any correct inserts.

5.4 Coexpression of VEGF-C with helper proteins

The two expression plasmids for calreticulin and cyclophilin B were successfully transfected into the VEGF-C-expressing Sf9 cell line. The expression vector pIZ-HygroA was also transfected to the same cells to be used as a negative control. In addition to these double-transfectants, pIZ-HygroA-hCALR-V5-H6, pIZ-HygroA-hPPIB-V5-H6 and pIZ-HygroA, VEGF-C-expressing Sf9 cells and wild type Sf9 cells were grown to use as controls. All cells, except the wild type Sf9 cells, were grown under antibiotic selection with 50 µg/ml zeocin to maintain VEGF-C expression. The double transfectants were additionally selected with 10 or 20 µg/ml hygromycin B. Irrespectively of the hygromycin concentration used, cells transfected with pIZ-HygroA, pIZ-HygroA-hCALR-V5-H6 or pIZ-HygroA-hPPIB-V5-H6 rapidly started to tolerate the hygromycin B after the initial selection crisis, while other cells died within three days from the addition of the hygromycin B.

5.5 Western Blot: Detection of helper proteins and VEGF-C

Conditioned cell culture supernatants and lysates were harvested from cultures of double transfectant Sf9 cells. VEGF-C could be identified in all samples except in the wild type cells as a major band of approximately 30 kDa (Figure 5). Minor bands were visible for some samples at around 15 kDa and 45-55 kDa. No signal was seen for the sample of wild type Sf9 cells (which do not express VEGF-C). VEGF-C-expressing Sf9 cells, which had not been transfected with pIZ-HygroA or its derivatives showed the strongest signal for VEGF-C.

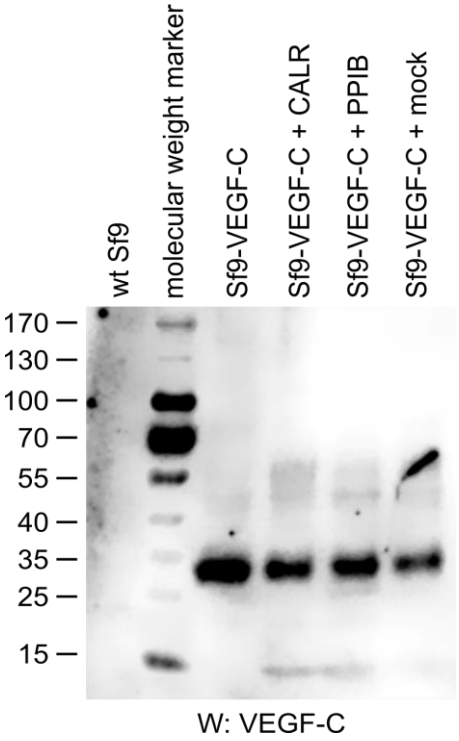

Figure 5. Detection of VEGF-C. Western Blot of Sf9 cell culture supernatants (detection with anti-VEGF-C antibody AF752 (R&D Systems), reducing samples, 4-20% SDS-PAGE).

On the non-reduced Western blot two bands of approximately 50-55 kDa and 100-110 kDa can be observed (Figure 6), corresponding to the covalently linked dimer and the non-covalently linked dimer. The non-covalently linked dimer represents the majority of the signal. As already seen in the reducing SDS-PAGE, the cells which express only VEGF-C appear to produce more VEGF-C compared to the double transfectants. This effect was unrelated to the expression of the helper proteins as the mock transfectant (empty pIZ-HygroA plasmid without any helper protein cDNA insert) showed lower expression levels of VEGF-C as well.

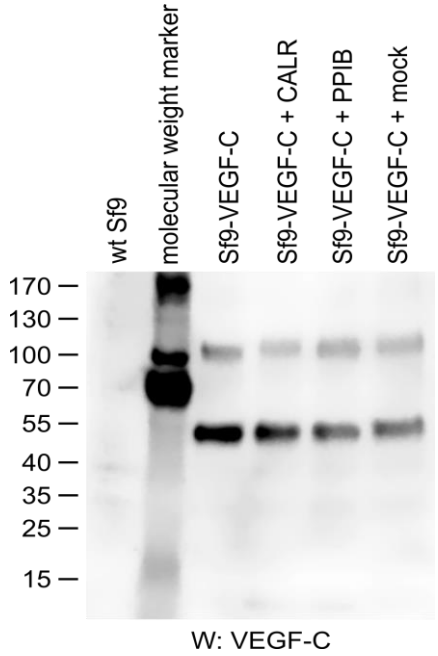

Figure 6. Detection of VEGF-C. Western Blot of Sf9 cell culture supernatants, detection with anti-VEGF-C antibody AF752 (R&D Systems), non-reduced samples, 4-20% SDS-PAGE.

In the Western blot of the cell lysates, the V5-tagged calreticulin was detected as a band of 75-80 kDa and the V5-tagged cyclophilin B as a band of approximately 35 kDa. The loading control β-actin appeared as a weak band of approximately 55 kDa (Figure 7). For the helper proteins, the signal from cyclophilin B appears to be slightly stronger than the one for calreticulin indicating stronger expression of the cyclophilin B. The β-actin signal in the Sf9-VEGF-C+CALR sample is also slightly weaker, which probably reflects the slower growth of the cells transfected with the CALR expression vector. In all other samples, β-actin was present at roughly similar levels. The zigzag line seen in the 700 nm channel was likely caused by the migration front of the bromophenol blue dye, which is a component of the standard Laemmli SDS-Page loading buffer.

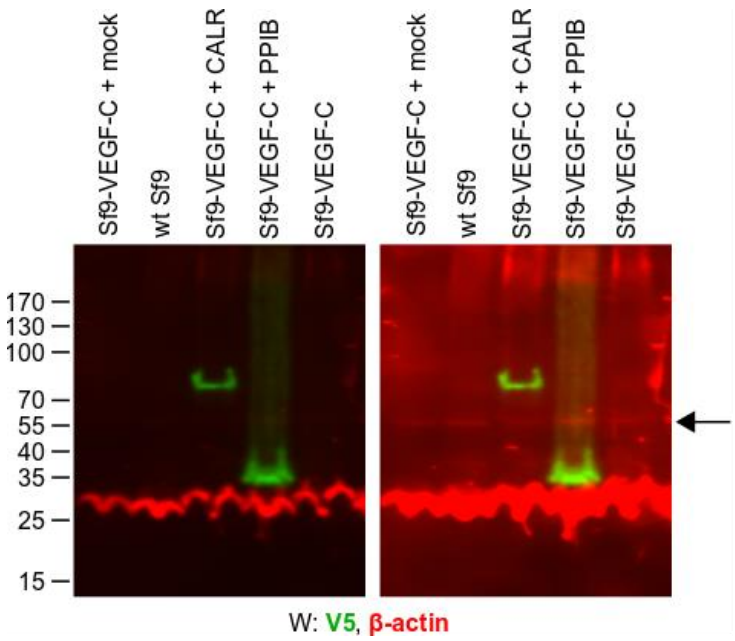

Figure 7. Detection of the V5 tag and β-actin. V5-tagged helper proteins are shown in green and β-actin (black arrow) in red. 4-20% reducing SDS-PAGE. Two different exposures are shown due to the vastly different signal-to-noise ratio between V5- and β-actin channels.

5.6 Ba/F3-VEGFR-3/EpoR assay

When harvesting the cell culture supernatant for the Ba/F3-VEGFR-3/EpoR assay, it was noted upon visual inspection that the cell densities of the cell lines Sf9-VEGF-C+ CALR and Sf9-VEGF-C were significantly lower compared to the other cell lines.

A clear dose-dependent response was only seen for the positive control (Figure 8). No VEGF-C-mediated survival effect could be detected for conditioned medium from the Sf9+VEGF-C and Sf9+VEGF-C + CALR cell lines as the response was indistinguishable from non-VEGF-C-expressing Sf9 cells. The Sf9+VEGF-C + PPIB cell line was the only one of the samples where a difference in the growth of the Ba/F3 cells was observed. However, the effect remained modest and was not able to completely offset the growth-inhibiting effect of the insect cell culture medium at higher concentrations.

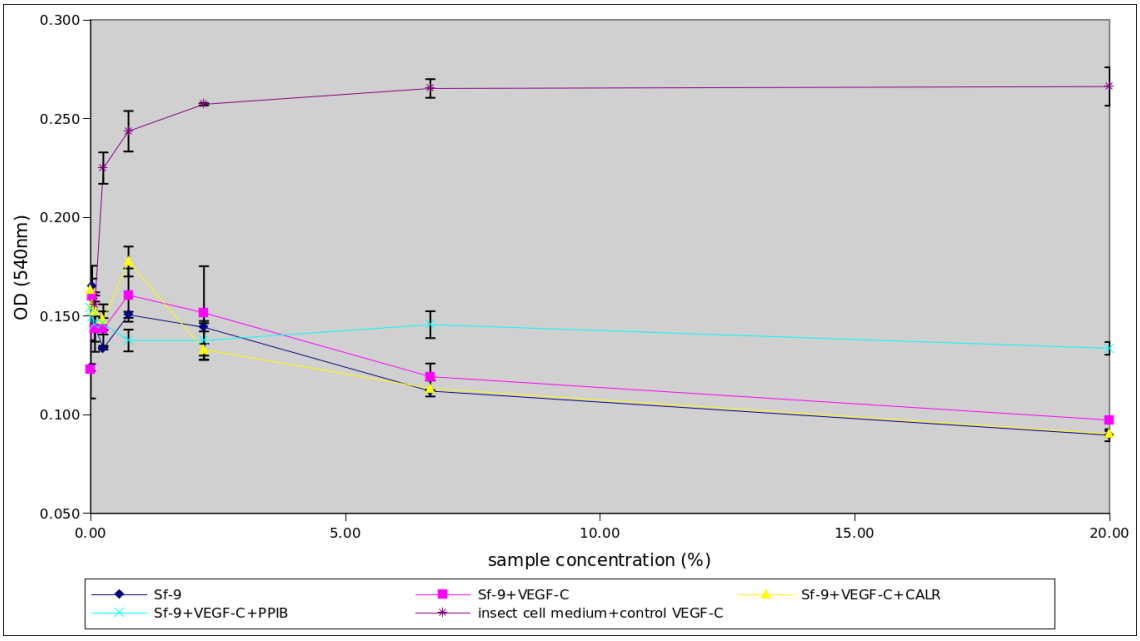

Figure 8. Ba/F3-VEGFR-3/EpoR assay. Only the positive control showed a clear dose-dependent response. For Cyclophilin B, a positive effect on VEGF-C activity could be detected, while the calreticulin response was indistinguishable from the negative controls. The cyclophilin B effect, however, was very modest and did not compensate for the growth-inhibiting effect of the insect cell culture medium on the Ba/F3-VEGFR-3/EpoR cells.

6 Discussion and Conclusions

6.1 Quality improvement of VEGF-C

The idea of this thesis project was to examine the effects of introducing different helper protein cDNAs into an insect cell expression system. The aim was to improve the folding of the recombinant pro-VEGF-C produced by these cells. All of the five chosen helper proteins work as chaperones or isomerases in the biosynthesis of proteins. Each of them is expressed by the insect cells but in more moderate amounts than in mammalian cells. Theoretically, increasing the amount of these helper proteins would improve the folding of the pro-VEGF-C.

Two of the helper proteins together with VEGF-C were successfully introduced into the Sf9 cell line. However, it could not be shown that either had a meaningful impact on the quality of the produced VEGF-C. For calreticulin, no effect could be detected, while for cyclophilin B, a modest effect was observed in the Ba/F3-VEGFR-3 assay.

In the Western blots for the cells coexpressing both VEGF-C and calreticulin or cyclophilin B, no changes could be seen in the ratio of the covalently linked to the noncovalently linked VEGF-C dimers. However, the expression levels of VEGF-C were clearly lower in the double transfectants than in the cells expressing only VEGF-C. This was independent of the introduced cDNA: also cells transfected with the empty pIZ-HygroA plasmid showed this reduction of VEGF-C expression levels. Conclusions whether there was any effect in the folding of VEGF-C cannot be made based on these Western blots. The prolonged growth of the cells during the selection with hygromycin (4-5 weeks) was likely not responsible for the decline of the VEGF-C expression levels since the control cells (Sf9-VEGF-C) were kept in culture for the same period of time and they did not show the same decline. A possible explanation is a general suppressing effect of hygromycin on protein translation (Borovinskaya et al., 2008). However, this would result in overall reduced cell proliferation. In fact, the VEGF-C+CALR double transfectants did grow slower than the other cells, but in the two other double transfected cell lines, this effect was not seen.

Additional experiments are required to confirm the modest effect that was detected for PPIB in the Ba/F3-VEGFR-3/EpoR assay. Three factors were limiting the sensitivity in this assay:

1. The maximal response of the Ba/F3-VEGFR-3/EpoR cells to VEGF-C control was not optimal and reached only about 25% of the ideal values indicating that the viability of the cells was not optimal, perhaps due to prolonged culture. This was also seen when the cells were counted, as only about 58% of all cells were alive at the beginning of the assay. The viability of stably transfected Ba/F3-VEGFR-3/EpoR cells should preferably be above 90% in order to get a strong proliferation response.
2. The second reason for the low responses to the VEGF-C-conditioned media is the fact that the Sf9 cells produced pro-VEGF-C, which is an inactive precursor of VEGF-C. In the Ba/F3-VEGFR-3/EpoR assay, the endogenous activation of pro-VEGF-C was left to the Sf9 cells (during production) and Ba/F3-VEGFR-3/EpoR (during the assay itself). However, the low response of the cell culture supernatant when compared with the positive control shows that the activation of pro-VEGF-C by secreted proteases of Sf9 and Ba/F3 cells is very limited. It is unknown whether the VEGFR-3/EpoR chimeric receptor that is used in the Ba/F3 assay is subject to the same competitive inhibition by pro-VEGF-C as the native VEGFR-3. If so, this might have additionally contributed to the low re-

sponse even though pro-VEGF-C has a much lower affinity towards VEGFR-3 compared to mature, active form. In order to improve this assay, an activation step should be included, for example by limited exposure of the samples to trypsin or proteinase K.

3. Insect cell media negatively affects the growth of Ba/F3 cells (conditioned insect cell media has a pH between 6 and 6.5). This effect was visible already at a concentration of less than 10% (curve for Sf9 cells in Figure 8). This limited the maximal sample amounts that could be used, and higher concentrations of the conditioned insect cell media would likely not give a better response. One way to increase the concentration of insect cell media without negatively affecting Ba/F3 growth would be to dialyze the conditioned insect cell media against DMEM prior to the Ba/F3 assay.

6.2 Western blotting

In the reduced samples (Figure 5), all the disulfide bonds of VEGF-C have been broken with 2-mercaptoethanol into a double band of 29/31 kDa. However, only a single band was detected. This is likely due to the presence of the C-terminal V5-His tag, which increases the size of the 29 kDa C-terminal domain of VEGF-C by approximately 2 kDa.

In the non-reduced samples (Figure 6) the expected sizes for the VEGF-C bands are around 58 kDa and 110-120 kDa. The 58 kDa band is monomeric VEGF-C, in which the C-terminal domain of VEGF-C is covalently linked to the rest of VEGF-C via disulfide bonds. The 110-120 kDa band results likely from a small fraction of VEGF-C, in which the central VEGF-C homology domains of the dimer are covalently linked. Both of these bands appear slightly smaller than expected. This presumably results from the fact that these polypeptides do not migrate as elongated polypeptides chains (like the molecular weight marker does). Instead, the cystine knot structure and the additional cysteine bridges in the C-terminal domain likely give the molecule a more compact shape despite reduction and the presence of SDS.

6.3 Cloning

Regardless of several attempts with different conditions the product yield of most PCRs remained low. Therefore traditional restriction enzyme cloning proved difficult and TOPO cloning was used. The TOPO cloning, however, did not yield as many colonies as expected (several instead of several hundreds), presumably due to the age of the kit or inappropriate storage. Calreticulin and cyclophilin B were successfully cloned via the TOPO intermediates into the expression vector. TOPO T/A cloning of CDC37 was later also successful after the correct template was received from the Genome Biology Unit. However, no further experiments could be conducted with it within this thesis project.

VEGF-C contains an extraordinary amount of cysteine residues, most of which form disulfide bonds. Therefore, P4HB and PDIA3 were perhaps the most interesting helper proteins; however, their clonings could not be completed within this thesis project and their effects on the quality of recombinant VEGF-C produced by insect cells cannot be reported. DNA plasmids isolated from colonies that were obtained from the TOPO T/A cloning of PDIA3 did not contain any inserts. Also no PCR product with Phusion HF was gained for PDIA3 to be used in TOPO Blunt cloning. Colonies obtained from the TOPO cloning of P4HB were shown by DNA sequencing to contain CDC37 inserts instead, which might be due to a mix-up of samples at some stage.

6.4 Lowered VEGF-C expression

Originally, co-expression of VEGF-C with the helper proteins was planned for both Sf9 and High Five (HF) cells. However, Western blots revealed that the HF cells had almost completely lost their VEGF-C expression during the hygromycin selection and were therefore not analyzed. The VEGF-C expression may have faded because the cells had been maintained for too long in culture. Another possible reason could be an effect of the newly created hygromycin selection vector or a direct effect of hygromycin on the cells. Loss of expression despite continued antibiotic selection by epigenetic mechanisms is not unknown (Wolffe and Matzke, 1999). The VEGF-C expression in both Sf9 and HF cells had been established with the InsectSelect expression system (Invitrogen), but it had not been tested whether these cell lines would maintain VEGF-C expression upon prolonged culture.

If prolonged culture was the reason of the silencing of VEGF-C expression, a new cell line could be created, which expresses both VEGF-C and the helper protein from the

same DNA construct. Thereby silencing of the VEGF-C expression cassette due to prolonged culture could be avoided. However, trying different combinations of the five helper proteins would be difficult with such a system.

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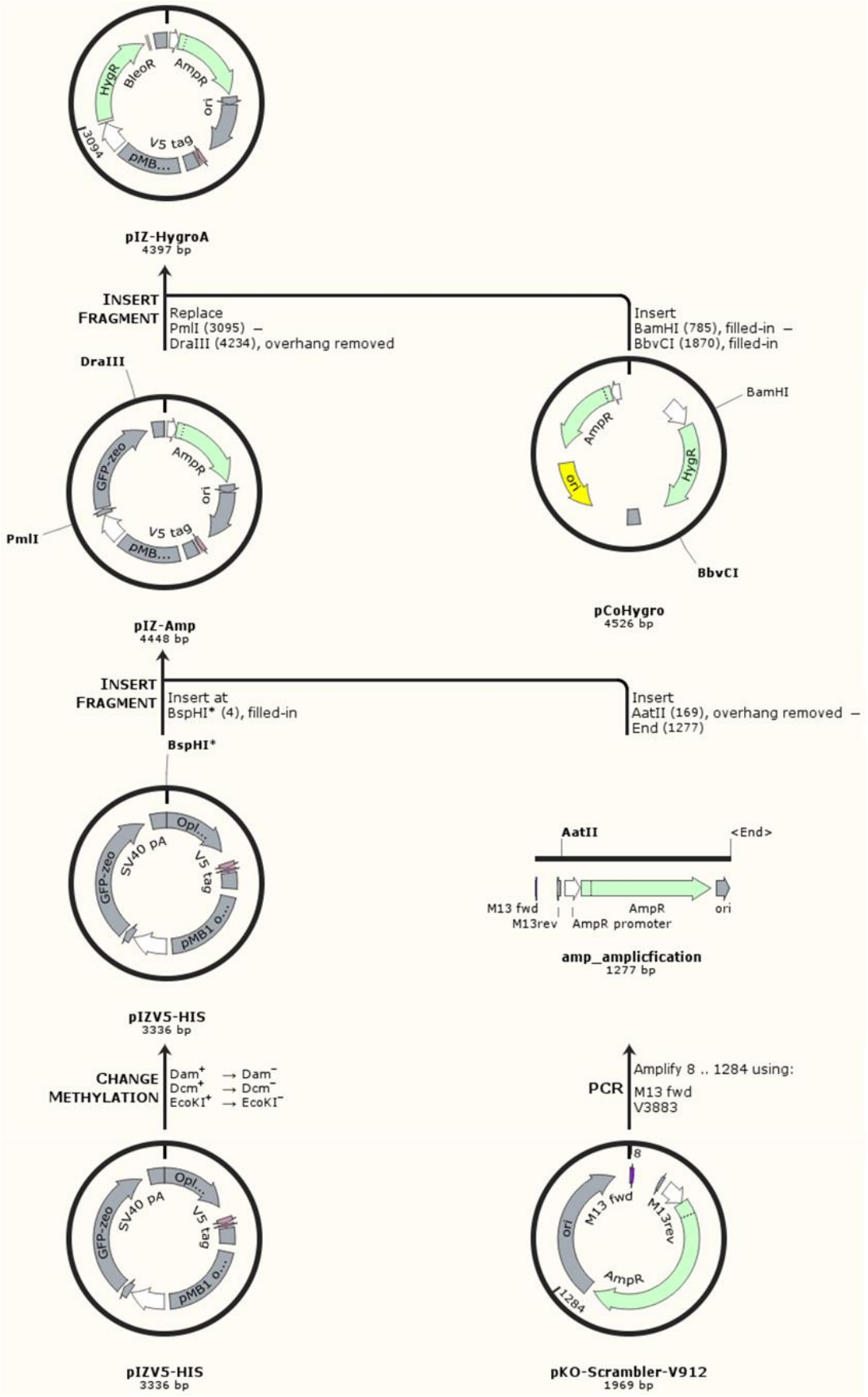
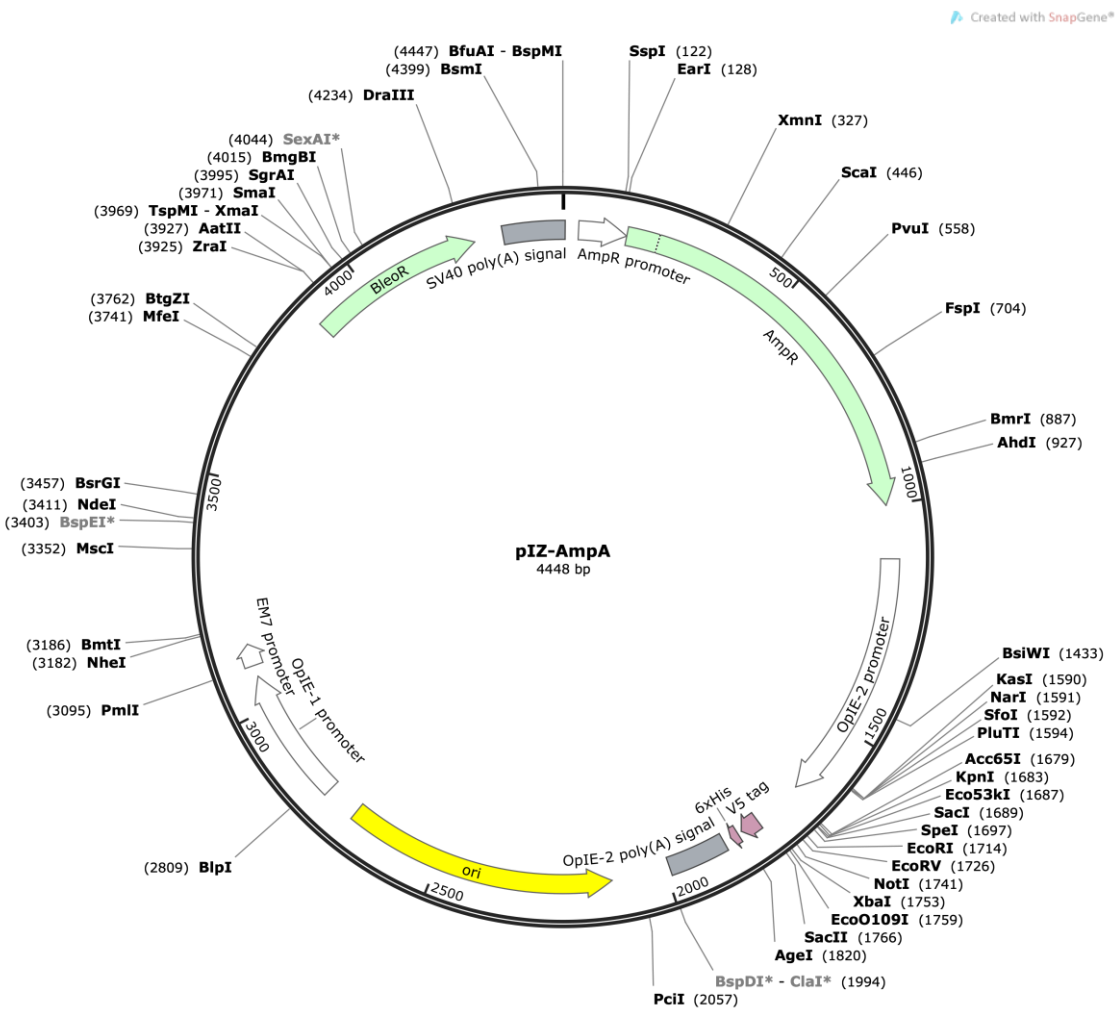
Appendix 1. Cloning history of the expression vector pIZ-HygroA



Appendix 2. pIZ-AmpA



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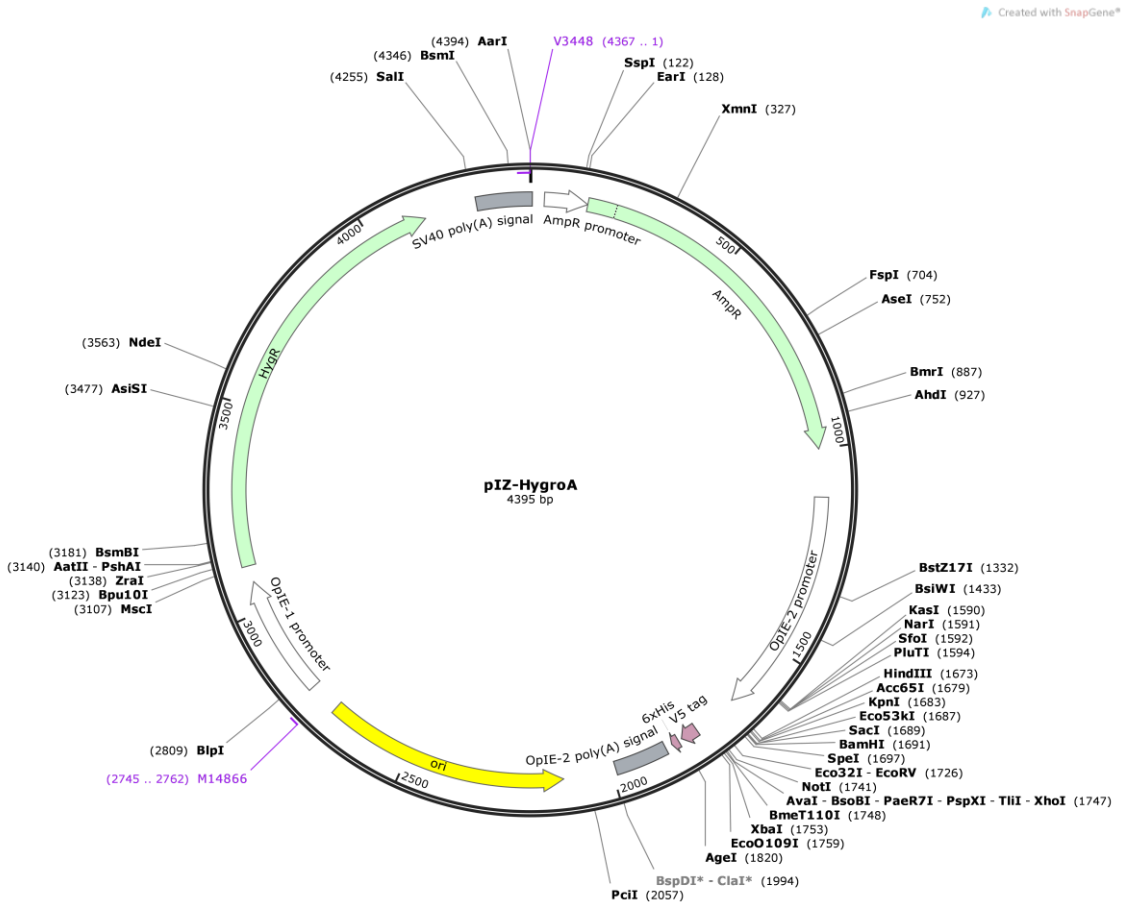
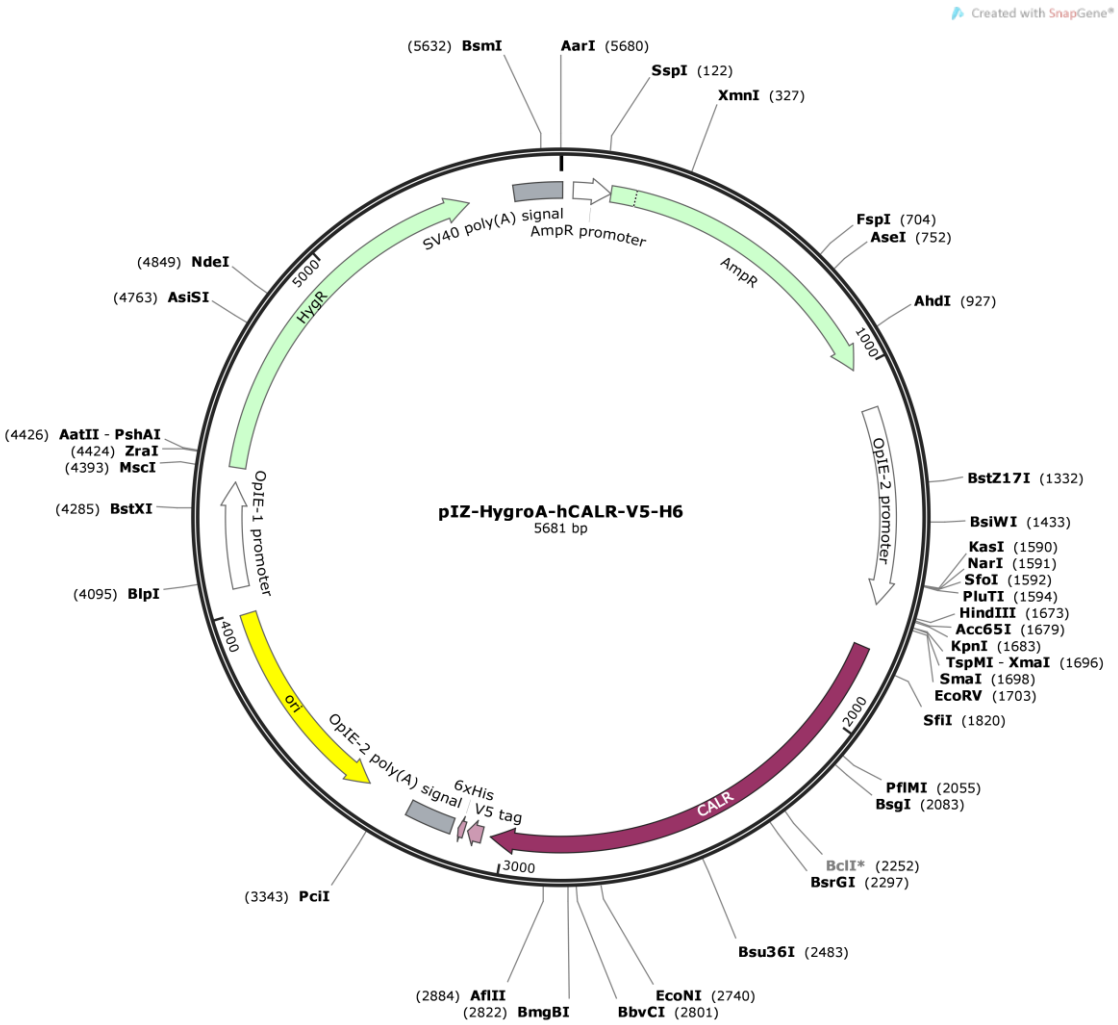


Appendix 4. pIZ-HygroA-hCALR-V5-H6



Appendix 5. pIZ-HygroA-hPPIB-V5-H6